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Phylogenetic analysis of mt COⅠ reveals the cryptic lineages in *Phenacoccus solenopsis* complex (Hemiptera: Pseudococcidae)

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Abstract: The mealybug species, *Phenacoccus solenopsis* Tinsley has caused severe economic losses during the last two decades in many countries. Recently this alien invasive species has been introduced in China and threatened the agricultural production. The rapid and accurate identification of the species has been the basis of the scientific research and management of the alien invasive species (IAS). In this study, the mitochondrial cytochrome oxidase subunit I (mt COⅠ) of the *P. solenopsis* materials collected from Sanya City and Lingshui County, Hainan Province were sequenced and compared with the known sequences of the species from Florida of the USA. We unexpectedly found that there were two divergent clades in the mealybug species, *P. solenopsis*, based on the phylogenetic tree. The genetic distance between the collections from China and those from the USA was approximately 3%. This was the first report on the molecular identification of *P. solenopsis* in China based on the mt COⅠ marker. These data suggest that *P. solenopsis* might be a species complex which includes at least two cryptic lineages or sibling species. The newly collected *P. solenopsis* materials from Sanya and Lingshui are of a cryptic lineage or species in the complex. The data also suggest the source of Sanya and Lingshui populations of *P. solenopsis* in the present study might not come from Florida of the USA. The discovery of the cryptic lineages in *P. solenopsis* complex is helpful for its accurate identification and the future research in its invasion ecology.

Key words: *Phenacoccus solenopsis*; biological invasion; species complex; cryptic lineages; mt COⅠ marker; phylogenetic analysis

1 INTRODUCTION

Biological invasions have been one of the important focuses of management and research activities worldwide during the last two decades because of their ecological and economic effects on the invaded regions or countries. The rapid and accurate identification of the alien invasive species (IAS) has been the basis of the scientific research and management of the IAS. However, the number of the IAS on the invaded habitats might be overlooked or underestimated because of the existence of the sibling species (McDonald and Koehn, 1988; Geller *et al.*, 1994, 1997; Genner *et al.*, 2004; Chu *et al.*, 2006) or genetic lineages (Saltonstall, 2002), which are morphologically indistinguishable but genetically distinct. The negative impact of the IAS was also often underestimated because the sibling species or genetic lineages often have different ecological characteristics or spread pattern (Geller *et al.*, 1997; Saltonstall, 2002; Genner *et al.*, 2004). The mitochondrial cytochrome oxidase subunit I (mt COⅠ) has been proposed as the DNA barcoding of species and there is a growing literature demonstrating that mt COⅠ will reliably discriminate a diverse range of taxa at the species level (Hebert *et al.*, 2003a, 2003b). The marker has the potential to be adopted globally for the rapid and accurate identification of the IAS (Armstrong and Ball, 2005).

The mealybug species, *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: CocCOⅠdea: Pseudococcidae), in the USA was first described by Tinsley (1898). In 1991, this species was found to infest cultivated cotton in the USA (Fuchs *et al.*, 1991). Subsequent reports showed that *P. solenopsis* distributed widely in the Central America, the Caribbean, Ecuador, Chile, Argentina and Brazil (Culik and Gullan, 2005; Nagrare *et al.*, 2009). Recent reports showed that *P. solenopsis* has been

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introduced into many Asian countries including Pakistan, India (Hodgson *et al.*, 2008; Nagrare *et al.*, 2009) and Nigeria (Akintola and Ande, 2008). This species has been considered as invasive species in Asian though the accurate source has not been determined or known. In China, *P. solenopsis* was first found in Guangzhou of Guangdong Province in 2008 (Wu and Zhang, 2009). Subsequent monitoring showed that the species was also found in Sanya City (Xu and Fu, personal communications) and Lingshui County (this study), Hainan Province.

The mealybug could cause serious economic losses in the invaded countries or regions (Lu *et al.*, 2008; Wu and Zhang, 2009). For example, the cost of the pesticide used to control the pest was US $ 121.4 million in Punjab of Pakistan during two months in 2007 (Hodgson *et al.*, 2008; Wu and Zhang, 2009). The total damage in 2007 was estimated to range from US $ 400 000 to 500 000 in north India alone (Nagrare *et al.*, 2009). In addition, the host range of *P. solenopsis* includes several ornamental plants, fruit crops, vegetables and field crops belonging to at least 14 families in the USA (Nagrare *et al.*, 2009). Based on the international pest risk analysis method by Wang *et al.* (2009), *P. solenopsis* is high risk invasive species to China. The mealybug could occur in most areas of 17 provinces and also in part of the other 11 provinces (Wang *et al.*, 2009). Owing to its potential damages and rapid spread of the alien species, the mealybug has been considered as an important quarantine pest in China in 2009 (http://www.aqsiq.gov.cn/qyhypd/ncp/zwjajy/jjzwjyjy/200902/t20090212_105809.htm). Until now, the identification of this species in China has mainly depended on the morphological characteristics. However, the Chinese population of the species has not been identified based on the molecular marker.

The objective of the present study is to sequence mt COⅠ of the *P. solenopsis* from Sanya City and Lingshui County, Hainan Province of China and to compare the sequences with the GenBank sequences from the USA. We unexpectedly found that there were two divergent clades in the mealybug species, *P. solenopsis* based on the analysis of mt COⅠ marker. Finally, the invasive pathways of the mealybug and future researches are discussed.

2 MATERIALS AND METHODS

2.1 Sample collection and identification based on morphological characteristics

The mealybug samples collected in Sanya City and Lingshui County, Hainan Province in March and May, 2009, respectively. And the samples were put into the tube containing 95% ethonal and stored at -20℃. The species identity of these collections was first identified based on the morphological characteristics using the methods described by Fu *et al.* (2009). Then the determined species identity was further identified by Professor Wu San-An (Beijing Forestry University) and Dr. Douglass R. Miller (Systematic Entomology Laboratory, Agricultural Research Service, U. S. Department of Agriculture, and Department of Entomology, National Museum of Natural History, Smithsonian Institution).

2.2 DNA extraction, the polymerase chain reaction and sequencing

A single individual mealybug was subjected to lysis and DNA extraction followed the procedure of Frohlich *et al.* (1999). The polymerase chain reaction (PCR) was employed to amplify fragment of the mt COⅠ gene (800 - 820 bp), using parameters and PCR primers described by Frohlich *et al.* (1999). PCR assays were conducted using 2 uL of each template DNA in a total reaction volume of 25 uL. The PCR reaction mix and PCR conditions followed Frohlich *et al.* (1999). PCR products were separated on 1.0% agarose gels, and bands were visualized by ethidium bromide staining and viewed with a UV light source. PCR products were purified using a kit according to the manufacturer's instructions. The DNA sequence for each PCR product was determined from both the 5′end and the 3′ end at the High-Tech Research Center, Shandong Academy of Agricultural Sciences. Five individuals from Sanya collection and two individuals from Lingshui collection have been used to obtain the mt COⅠ in the present study.

2.3 Phylogenetic analysis of *P. solenopsis* and nucleotide divergence estimates

The mt COⅠ sequences of the *P. solenopsis* collections from Hainan were determined (Code: Sanya1 - Sanya5, Lingshui1 - Lingshui2), and reference mt COⅠ sequences of *P. solenopsis* from Florida of the US (Code: EU267208USA - EU267212USA) were obtained from the GenBank database. The *P. solenopsis* from Florida of the US has also been identified by Dr. Douglass R. Miller (Systematic Entomology Laboratory, Agricultural Research Service, US Department of Agriculture and Department of Entomology, Smithsonian Institution) (Judith K. Brown, personal communication). The mt COⅠ sequences of the other mealybug species in the GenBank were used as outgroups: *Phenacoccus manihoti* (EU267196P. manihoti), *P. pergandei* (AB439518P. pergandei), *Ferrisia virgata* (EU267205F. virgata), *Ferrisia gilli* (EU267203F. gilli), *Dysmicoccus brevipes* (EU267214D. brevipes), *Dysmicoccus neobrevipes* (EU267213D. neobrevipes), *Palmicultor palmarum* (EU267218P. palmarum), *Hypogeococcus pungens* (EU267216H. pungens).

These mt COⅠ sequences were aligned using the CLUSTAL W algorithm (Thompson *et al.*, 1994). Distances were calculated with the Kimura 2-parameter model of MEGA2.1 (Kumar *et al.*, 2001). The MP (Maximum Parsimony) and UPGMA (Unweighted Pair Group Method with Arithmatic Mean) algorithms available in MEGA2.1 were used to infer phylogenetic relationships, respectively. One thousand bootstrap replicates were performed for each analysis.

3 RESULTS

3.1 Phylogenetic analysis of the *P. solenopsis* mt CO I

The mt COⅠ sequences of the mealybugs from Sanya City and Lingshui County were edited, which yielded a ~710-base fragment for each *P. solenopsis* mt COⅠ sequence. Only one of the mt COⅠ sequences determined from Sanya City has been deposited in GenBank because all of the 5 mt COⅠ sequences were identical and the accession number is GQ398777. The 2 mt COⅠ sequences from Lingshui County were deposited in GenBank and their accession numbers are GQ903580 and GQ903581, respectively.

The mt COⅠ MP (Fig. 1) and UPGMA (not shown) trees revealed similar results and two main collections from*P. solenopsis* were supported by robust bootstrap value, respectively. Two distinct clades were revealed with two major nodes strongly supported by 99% bootstrap values. One major clade (Florida clade) contained sequences of *P. solenopsis* from Florida of the US (EU267208USA - EU267212USA). The other major clade (Hainan clade) contained sequences of *P. solenopsis* from Sanya City and Lingshui County, Hainan Province of China.

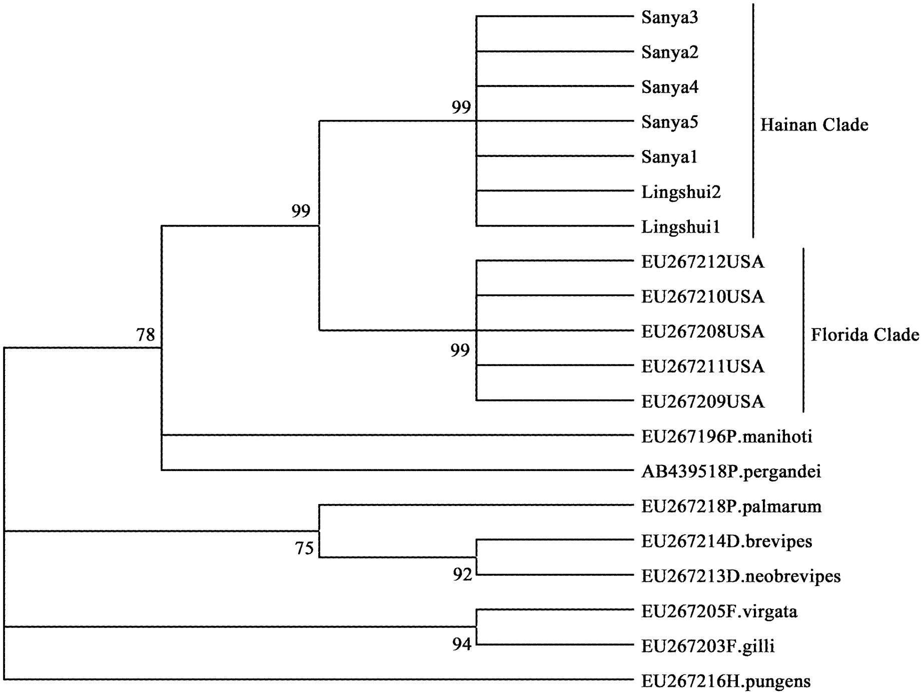

Fig. 1 The MP tree for *Phenacoccus solenopsis* based on a mt COⅠ fragment (~710 bases) (1 000 bootstrap iterations; >70% cutoff)

3.2 Nucleotide divergence estimates

Within and between nucleotide sequence divergences were calculated for the two collections from Hainan Province of China and from Florida of the US. The nucleotide sequence divergences within each collection are both approximately zero. However, the nucleotide sequence divergence between them was approximately 3%. The genetic distance between the *P. solenopsis* mt COⅠ sequence (from either Hainan Province of China or Florida of the US) and the other mealybug mt COⅠ sequences was more than 13%.

4 DISCUSSION

According to our knowledge, this is the first report on the molecular identification of *P. solenopsis* in China based on the mt COⅠ marker. Phylogenetic tree showed that there were two divergent clades in this mealybug species (Fig. 1). The genetic distance between the collections from China and those from the US in the present study was approximately 3%. The research in Hebert *et al.* (2003a) showed that intraspecific mitochondrial divergence in most of species does not exceed 2% based on mtⅠ sequence.

Thus, *P. solenopsis* might be a species complex which might include at least two cryptic lineages or sibling species.

Current data showed that the morphology of adult female *P. solenopsis* from Guangzhou of China was identical to that from Taiwan of China, Pakistan, and Thailand. However, the morphological characteristics of the adult female of these samples were somewhat different from those from America. The multilocular disc pores and tubular ducts on the ventral submargin of the abdomen in adult female *P. solenopsis* from America continent were rare, even absent (Wu and Zhang, 2009). In our study, the morphology of adult female *P. solenopsis* from Sanya City and Lingshui County was identical to the description of the species from Guangzhou and Taiwan of China, Pakistan, and Thailand. Thus, whether the morphological difference of the geographical population was consistent with their genetic differentiation should be further studied.

Our data also suggest the source of Sanya and Lingshui populations of *P. solenopsis* in the present study might not come from Florida of the US. In the future work more samples should be collected and to analyze the genetic differentiation and genetic structure of the species. Those studies will help to understand the invasion history, invasion pattern and source of the invaded population in China. This ecological information may help us to cut off the invasion pathway or to introduce the biological control agents or to learn from the measures taken by the other countries in the world.

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线粒体细胞色素氧化酶 I（mt COI）序列分析揭示扶桑绵粉蚧复合种存在隐存谱系

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摘要：过去约 20 年间扶桑绵粉蚧 *Phenacoccus solenopsis* Tinsley 在许多国家造成严重危害。近年来该害虫传入我国，对农业生产构成严峻威胁。外来入侵物种的快速准确鉴定是科学研究及其管理的基础。本研究对来自海南三亚和陵水地区的扶桑绵粉蚧的线粒体细胞色素氧化酶 I（mt COI）测序并与美国佛罗里达州的扶桑绵粉蚧序列进行了比较。系统发育树表明该物种存在两个遗传支系，即海南支系与佛罗里达支系；这两个支系的遗传距离约 3%。这是利用 mt COI 分子标记对我国扶桑绵粉蚧的首次分子鉴定。这些结果提示：(1)扶桑绵粉蚧可能是至少含有两个隐存谱系或姊妹种的复合种；(2)新入侵我国海南三亚和陵水地区的扶桑绵粉蚧是该复合种内的一个隐存谱系或物种，且海南三亚和陵水种群可能不是来自美国佛罗里达州。扶桑绵粉蚧隐存谱系的发现对于该物种的分子鉴定及其入侵生态学研究具有重要意义。

关键词：扶桑绵粉蚧；生物入侵；复合种；隐存谱系；mt COI；系统发育分析

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